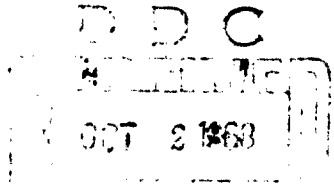
TRANSLATION NO. 2215

DATE: March 1968



DEPARTMENT OF THE ARMY
Fort Detrick
Frederick, Maryland

CLOSTRIDIUM BOTULINUM F
Report IV

Obtaining Rabbit Antibotulinum Serum*

[Following is the translation of an article by I.M. Mikhaylova, Moscow Institute of Vaccines and Sera imeni Mechnikova, published in the Russian-language periodical Zhurnal Mikrobiologii, Epidemiologii i Immunobiologii (Journal of Microbiology, Epidemiology and Immunobiology) No. 11, 1966, pages 56-62. It was submitted on 9 March 1966. Translation performed by Sp/7 Charles T. Ostertag, Jr.]

*Report I. Zh. mikrobiol., 1965, No. 6, p 101; Report II, Ibid., No. 10, p 39.

As is known, at the present time the specific agents for the treatment of botulism are hyperimmune horse antitoxic sera. But up until now the literature has not contained any information on the obtaining of type F antitoxic serum for medicinal and diagnostic purposes by means of the immunization or hyperimmunization of other animals. Dolman and Murakami (1961) obtained type F rabbit serum, however it turned out to be weakly active and its strength was not determined, as this is taken for granted, in antitoxic units.

In the present work, for the purpose of obtaining hyperimmune type F rabbit serum we used botulinum toxoid type F, prepared by a method developed by us earlier (Mikhaylova, 1965). The toxoid was prepared from type F toxin, isolated by means of filtration of a 7-day culture of the botulism causative agent, cultivated in liver broth at 28°. The original toxin, containing 7000 Dlm in 1 ml, was poured into 4 bottles, into which a various amount of formalin (0.2, 0.4, 0.6, and 0.8%) was added. Rendering it harmless was carried out at 37°; all 4 series of toxoid contained 5 EC each in 1 ml (relative to 1 AU of standard antitoxin).

Each of the 4 series of toxoids, containing various concentrations of formalin, served as the antigen for the hyperimmunization of 5 chinchilla rabbits weighing 2-2.5 kg. The experiment was set up on 20 animals. All the rabbits were immunized over a period of 4 cycles. Each cycle consisted of 4 subcutaneous injections, which were carried out at 2-3 day intervals. Bleeding was carried out 7 days after the last injection. Two weeks after bleeding the following cycle of immunization was begun. In the first cycle the rabbits received successively 1, 1, 2, and 3 ml of toxoid, in the second - 1, 2, 2, and 3 ml, in the third -- 1, 2, 3, and 3 ml, and in the fourth - 1, 2, 3, and 4 ml. Thus, over the period of the 4 cycles each of the rabbits received 34 ml of toxoid.

From the rabbit blood, obtained after each bleeding, we prepared serum, which was drawn off into a separate test tube, added merthiolate (1:10 000) to it as a preservative, and stored it in a refrigerator at 4°. The sera, collected in various periods, was titrated parallelly with the working standard of type F antibotulinum serum, containing 30 AU in 1 ml (prepared at the State Control Institute imeni Tarasevicha). The antigen was concentrated glycerin botulinum toxin type F, containing 10 000 Dlm in 1 ml (prepared in the laboratory at the institute). A working dose of this toxin contained 100 Dlm. Initially we titrated a test dose of glycerin toxin with 1/5 AU of type F standard specific serum. After this we checked the neutralizing activity of the rabbit sera, and at the same time, as a control, we carried out the neutralization of a test dose of toxin with 1/5 AU of type F antibotulinum serum by intravenous administration to 4 mice weighing 16-18 grams. The animals were observed for 4 days. The titer was considered as the extreme dilution of serum, ensuring, in a mixture with the test dose of toxin, the survival of mice while 50% of the animals in the control died. The neutralization reaction of the botulinum toxin type F with the serum obtained was set up according to the instructions of the State Control Institute imeni Tarasevicha for determining the activity of antibotulinum sera of types A, B, C and E.

The results of titrating rabbit sera are presented in Table 1. As is seen in Table 1, the content of antitoxin in 1 ml of rabbit serum increased regularly with each cycle of immunization and the immunogenic properties of the toxoid did not depend on the concentration of formalin.

In the next series of tests we attempted to clear up if it is possible to obtain from rabbits type F antitoxins with a higher activity. For this purpose each of the above designated 4 groups was divided into 2 sub-groups. The animals of the 1st sub-group (3 rabbits) were immunized with toxin, containing 7000 Dlm in 1 ml over the period of the next 3 cycles. The method of administering the toxin and the intervals between injections, bleeding and rest were no different than those described above. In the first cycle the rabbits received 1, 1, 2 and 3 ml of toxin, in the second - 1, 2, 2 and 3, and in the third cycle - 1, 2, 3 and 3 ml, all told 25 ml of toxin each. The animals of the second sub-group (2 rabbits), which we considered as the control, continued to be immunized with the same toxoid for 3 cycles. In the fifth cycle they received 2, 2, 3 and 3 ml of toxoid, in the sixth - 2, 3, 3 and 4 ml, and in the seventh - 2, 3, 4 and 4 ml, all told 36 ml of toxoid each.

The results of hyperimmunization of rabbits with botulinum toxoids and toxins type F (Figure 1) showed that in rabbits, immunized with the type F toxin, the level of antitoxin in the blood after 3 cycles was increased significantly, while in the rabbits which continued to receive toxoid the titers of antitoxin in the blood increased significantly more slowly.

For the purpose of characterizing the rabbit sera obtained, which was taken both before and after hyperimmunization, we determined the content of protein fractions in them by the method of paper electrophoresis. Electrophoresis was carried out in an EFA-1 apparatus on chromatographic paper with the trademark "bystraya" /rapid/, which was cut into strips 40 cm in length and 2.5 cm wide. We placed 0.01 ml of serum on each strip. We used a veronal buffer, pH 8.6 and ionic strength equal to 0.05. The duration of electrophoresis was 18 hours with a potential gradient of 2.5 v/cm. The paper electrophoregrams, stained with an 0.05% acetic mercuric chloride solution of bromophenol blue, were cut and the elution of fractions was carried out in an 100 H. alkali solution. The percentage ratio of protein fractions was calculated on an electrophotocolorimeter.

In a comparison of the electrophoregrams of immune and nonimmune pigs no noticeable differences were detected (Figure 2). As can be seen in Table 2, during the process of immunizing the rabbits there was an increase in the content of proteins in the serum, due primarily to gamma- and beta-globulins, and the albumen content was reduced.

We also checked the resulting type F rabbit serum in the cross neutralization reaction with botulinum toxins type A, B, C, D, E and F, which were diluted to a content of 1 Dlm in 0.25 ml; the antibotulinum type F serum was diluted to a content of 100 AU in 0.25 ml, after which an equal amount of serum and toxin were mixed. The mixture was maintained for 45 minutes at room temperature and introduced intravenously in a volume of 0.5 ml to 4 mice, which we observed for a period of 6 days. For the purpose of control the mice were injected with toxin in quantities of 10, 2, 1 and 0.5 Dlm in 0.25 ml with 0.25 ml of a physiological solution of sodium chloride. The mice which received the mixture of type F serum with the toxins of type A, B, C and D died during the first days of observation, and those which received the mixture of type F serum and the toxins of type E and F remained alive.

While studying the antigenic structure of botulinum toxins type C alpha, C beta, and D, which have common components, Bulatova and Matveyev (1965) noted that in each of the strains studied there was a predominance of one main component, which determines the affiliation of the strain, and the remaining components were found in considerably lesser quantities. Being guided by the same principle as those authors in their study of the toxins of type C and D, we considered it interesting to determine the antigenic structure of the toxins of type F and E. In the test we used the botulinum toxins of type F and E (strains No. 153 and 188-20) and the strictly type specific sera of type F and E (to strains No. 153 and 188-20). The toxins for the reaction were prepared in the same manner as that previously described for glycerin concentrated botulinum toxin type F. All the stated toxins were diluted in such a manner that 1, 10 and 100 Dlm of toxin were contained in 0.25 ml of physiological solution. The toxins

were cross-mixed with antibotulinum sera of type F and E, and from these we prepared various dilutions, containing antitoxin in quantities ranging from 0.005 to 100 AU in 0.25 ml. The neutralization reaction was set up by the previously described method.

The results of numerous tests are summed up in Table 3, from which it can be seen that for the protection of mice from 1 Dlm of type E botulinum toxin (both strains) it required no less than 80 AU of antibotulinum serum type F, and from 1 Dlm of the type F toxin it required 4 (serum to strain No. 153) -30 (serum to strain No. 188-20) AU of type E serum. Based on the method of Bulatova and Matveyev, we calculated the multiplicity index - the relative number, representing the ratio of the amount of AU, necessary for the neutralization of toxin of a heterologous type, to the amount of AU, necessary for the neutralization of the same amount of toxin of a homologous type. This figure showed by how many times less it was necessary to take serum for the neutralization of a homologous toxin than for the neutralization of a heterologous toxin. It turned out that in the type F toxin the E component was found in almost a 100 time lesser amount than the same component (Figure 3). In the E toxin of both of the strains studied the ratio of E components was not the same: In the toxin of strain No. 153 there was half as much as in the toxin of strain No. 188-20, and both of these contained less than 1% of type F toxin.

It can be proposed that among the strains of the type E botulism microbes isolated up to the present time the type F microbes are found also, and though this problem requires a special study, the conclusion can be made that for the diagnosis of botulism it is advisable to also use the serum of type F along with the sera of types A, B, C and E.

In order to find out which common antigens the type F botulinum toxin has with the toxins of types A, B, C, D and E, which is shown in the specific precipitation reaction in agar by the method of Ouchterlony (1948), we used a 1% Difco agar on glass plates and dissolved in an 0.85% solution of sodium chloride. It turned out that the type F toxin had an antigenic relationship with the toxins of types A, B and to a lesser degree - with type D, forming intersecting precipitation lines with them, which spoke in favor of their non-identity (Figure 4). Such an antigenic relationship of the type F toxin with the toxins of types A, B and D, exposed only in the precipitation reaction in agar, requires further study.

Conclusions

1. During the hyperimmunization of rabbits with botulinum toxoid type F (for 4 cycles) and then with the type F toxin (for 3 cycles), a type F serum was obtained which had a titer of antitoxin, equaling on the average 115 AU. The titer of serum from rabbits in the control group, which received type F toxoid over a period of 7 cycles of hyperimmunization, equaled on the average 51 AU.

2. No noticeable difference was exposed in the mobility of the electrophoretic fractions in the immune and nonimmune rabbit sera. In the process of immunization the content of protein in the rabbit sera increased, due primarily to gamma- and beta-fractions.

3. In the neutralization reaction it developed that the antibotulinum serum type F, taken in a large quantity (80 AU), protected mice from 1 Dlm of type E boutlinum toxin. At the same time it was established that the type E and type F botulinum toxins have a mosaic structure - the type F toxin contained an insignificant amount of the E toxin component, and the type E toxin contains a small amount of type F toxin.

4. In the precipitation reaction in agar an antigenic bond was detected between the type F boutlinum toxin and toxins of types A, B and D.

5. In order to ensure the correct identification of the botulism causative agents, isolated from various objects (soil, food products, patients, carcasses, etc.), it is necessary to include type F serum along with the sera of types A, B, C, and E in the complex of diagnostic type specific antibotulinum toxins.

Literature

1. Bulatova, T.I., Matveyev, K.I., Zh. mikrobiol., 1965, No. 8, p 79.

2. Mikhaylova, I.M., Clostridium botulinum F. Report 2. Biochemical Properties. Study of Toxin and Toxoid Formation. Zh. mikrobiol., 1965, No. 10, p 39.

3. Dolman, S. Ye., Murakami, L., J. infect. Dis., 1961, v 109, p 107.

4. Ouchterlony, O., Acta path. Microbiol. Scand., 1948, v 25, p 186.

Table 1

Results of the hyperimmunization of rabbits with type F botulinum toxoids and toxins.

No. of rabbit	Content of formalin in toxoid (in %)	Titer of toxoid (in AU) based on the cycles of hyperimmunization									
		with Toxoid							with Toxin		
		1st	2nd	3rd	4th	5th	6th	7th	5th	6th	7th
1	0.2	1	8	55	65	65	65	65			
2		1	5	45	55	55	60	60			
3		1	2	10	15				35	45	60
4		1	5	25	35				90	130	200
5		1	5	15	20				55	65	75
6	0.4	1	5	45	50	50	55	55			
7		1	8	45	55	55	55	55			
8		1	5	20	25				55	65	75
9		1	2	15	20				50	60	70
10		1	5	25	30				80	110	125
11	0.6	1	5	25	30	30	30	35			
12		1	3	25	30	35	30	35			
13		1	5	30	40				75	85	95
14		1	2	10	15				30	35	45
15		1	10	60	70				150	250	400
16	0.8	1	8	50	65	65	70	70			
17		1	4	25	30	35	35	35			
18		1	3	15	20				35	40	50
19		1	5	35	40				80	90	100
20		1	5	25	30				70	80	90
Average titer		0.25	5	30	36.8	48.7	50	51.2	67.08	87.9	115.4

Table 2

Comparative content of proteins (in %) in various fractions of immune and nonimmune rabbit sera

Sera	Proteins	Albumins	Globulins		
			α	β	γ
Prior to onset of hyperimmunization	6.8	71.1	7.7	10.7	10.5
After the 7th cycle of hyperimmunization	7.8	51.4	11.9	17.8	18.9

Table 3

Cross neutralization reaction with the antitoxic antibotulinum sera of types F, E-153 and E-188-20, of the botulimum toxins of the same types and

Type of toxin	No of strain	Amount of AU, necessary for the neutralization of 1 Dlm of toxin with sera of various types		
		F	E-153	E-188-20
F	2901/470	0.01	4	30
E	153	80	0.02	0.02
	188-20	80	0.01	0.01

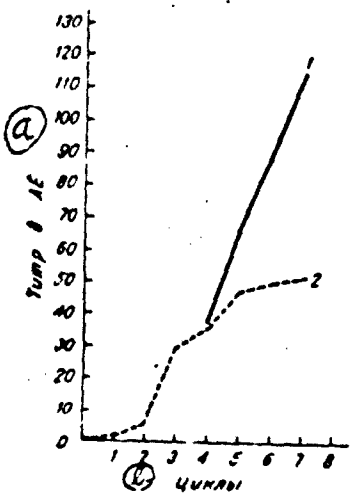


Figure 1. Growth curve for antibodies in the blood of rabbits, hyperimmunized with botulinum toxoids (1) and toxins type F (2).
a - titer in AU; b -cycles.

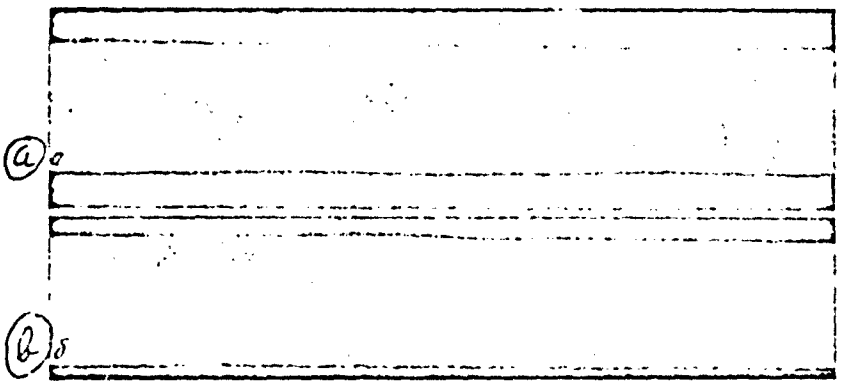


Figure 2. Electrophoregrams of rabbit sera prior to hyperimmunization (a) and after the 7th cycle of hyperimmunization (b).

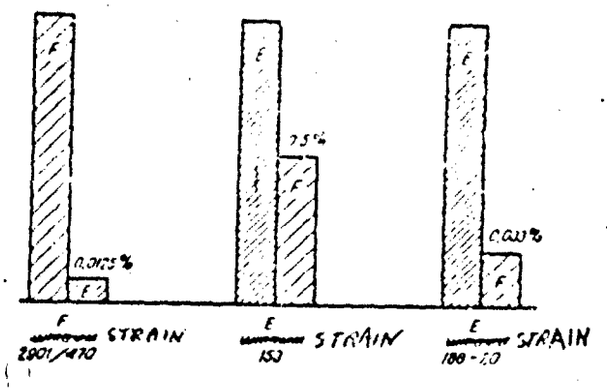


Figure 3. Antigenic structure of botulinum toxins, type F, E-153 and E-188-20 based on the data of the neutralization reaction.

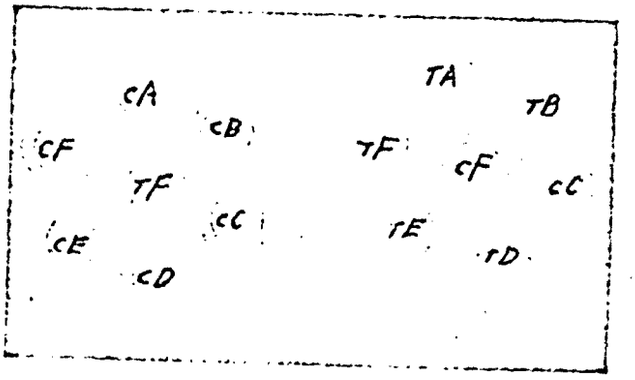


Figure 4. Precipitation reaction in agar between botulinum toxin (t) type F and antibotulinum sera (c) types A, B, C, D, E and F (left) and the precipitation reaction between antibotulinum serum type F and botulinum toxins type A, B, C, D, E and F (right)